IL-8 secreted in a macrophage migration-inhibitory factor- and CD74-dependent manner regulates B cell chronic lymphocytic leukemia survival

Inbal Binsky*, Michal Haran†, Diana Starlets*, Yael Gore*, Frida Lantner*, Nurit Harpaz†, Lin Leng‡, David M. Goldenberg§, Lev Shvidel†, Alain Berrebi†, Richard Bucala‡, and Idit Shachar*¶

*Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel; †Hematology Institute, Kaplan Medical Center, P.O. Box 1, Rehovot 76100, Israel; ‡Department of Medicine, Yale University School of Medicine, New Haven, CT 06520; and §Garden State Cancer Center, Center for Molecular Medicine and Immunology, Belleville, NJ 07109

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Chronic lymphocytic leukemia (CLL) is a malignant disease of small mature lymphocytes. Previous studies have shown that CLL B lymphocytes express relatively large amounts of CD74 mRNA relative to normal B cells. In the present study, we analyzed the molecular mechanism regulated by CD74 in B-CLL cells. The results presented here show that activation of cell-surface CD74, expressed at high levels from an early stage of the disease by its natural ligand, macrophage migration-inhibition factor (MIF), initiates a signaling cascade that contributes to tumor progression. This pathway induces NF-κB activation, resulting in the secretion of IL-8 which, in turn, promotes cell survival. Inhibition of this pathway leads to decreased cell survival. These findings could form the basis of unique therapeutic strategies aimed at blocking the CD74-induced, IL-8- dependent survival pathway.

apoptosis | invariant chain

B cell chronic lymphocytic leukemia (B-CLL) is characterized by the progressive accumulation of $CD5^+$ B lymphocytes in peripheral blood, lymphoid organs, and bone marrow (1). The hallmark of the disease is decreased apoptosis, resulting in accumulation of these malignant cells. Previous studies have shown that chronic lymphocytic leukemia (CLL) lymphocytes express relatively large amounts of mRNA for CD74, which is the cell-surface form of invariant chain (Ii), as compared with normal B cells (2, 3). CD74 is a nonpolymorphic type II integral membrane protein, which was originally thought to function mainly as an MHC class II chaperone (4). However, CD74 recently was found to play an additional role as an accessory-signaling molecule. In macrophages, CD74 demonstrates high-affinity binding to the proinflammatory cytokine, macrophage migration-inhibitory factor (MIF). MIF binds to the extracellular domain of CD74; this complex is required for MIF-mediated MAPK activation and cell proliferation (5). Moreover, the bacterium *Helicobacter pylori* was shown to bind to CD74 on gastric epithelial cells and to stimulate IL-8 production (6).

In a previous study, we showed that CD74 is involved directly in shaping the B cell repertoire (7, 8) by a pathway leading to the activation of transcription mediated by the NF-κB p65/RelA homodimer and its coactivator, TAFII105 (9). NF-κB activation is mediated by the cytosolic region of CD74 (CD74-ICD), which translocates to the cell nucleus (10). This signal is terminated by degradation of the active CD74-ICD fragment (11, 12). Moreover, we demonstrated recently that CD74 stimulation with an agonistic CD74 antibody leads to NF-κB activation, enabling entry of the stimulated B cells into the S phase, an increase in DNA synthesis, cell division, and augmented expression of members of the Bcl-2 protein family. Thus, these findings indicate that surface CD74 functions as a survival receptor (13).

In the present study, we sought to determine whether CD74 functions as a survival receptor in B-CLL cells. Our results show that MIF-induced CD74 activation initiates a signaling cascade that results in secretion of IL-8, which regulates B-CLL survival. Blocking this pathway induces cell death. Thus, CD74 expressed on the surface of B-CLL cells plays a critical role in regulating the survival of these malignant cells.

Results

CD74 Expression Is Highly Up-Regulated in B-CLL. To determine whether activation of the cell-surface protein CD74 in B-CLL cells leads to the induction of a signaling cascade that promotes the survival of these malignant cells, we first wished to determine the stage of the malignancy at which CD74 expression is up-regulated in B-CLL cells. Murine peripheral B cells (immature and mature populations) express cell-surface CD74 (13). We therefore used normal circulating mature B cells as a control for the malignant B-CLL cells, as was done in other studies of various aspects of CLL (14, 15). Purified B cells from healthy subjects as well as early- and advanced-stage B-CLL patients were analyzed by means of RT-PCR for the presence of CD74 mRNA (a segment common to all isoforms). As shown in Fig. 1*A*, low levels of CD74 were detected in normal B cells, whereas elevated levels of CD74 mRNA were observed in all of the CLL patients, regardless of the stage of disease [see supporting information (SI) Table 1]. To determine the levels of CD74 protein, lysates of normal B cells and early- and advanced-stage B-CLL cells were analyzed by Western blot analysis for CD74 expression. A significant elevation of the different CD74 bands corresponding to the various CD74 isoforms was detected in cells derived from B-CLL patients, regardless of disease stage (Fig. 1*B* and SI Table 1).

Next, we determined by flow cytometry whether these elevated levels of CD74 were expressed on the cell surface of B-CLL cells. As shown in Fig. 1*C*, compared with normal B cells, higher levels of CD74 were expressed on the cell surface of all B-CLL cells.

These results were uniformly seen in all of the samples examined regardless of the clinical parameters of the patients, including stage of the disease (either Rai or Binet), lymphocyte count, doubling time, previous administration of chemotherapy, type of chemotherapy, age, sex, morphology of the cells, Ig level, autoimmune phenomena, or CD38% and Zap-70 (see SI Tables 1 and 2).

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Conflict of interest statement: D.M.G. is a director and stockholder of Immunomedics, Inc., which is developing the hLL1 mAb.

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Abbreviations: CLL, chronic lymphocytic leukemia; B-CLL, B cell chronic lymphocytic leukemia; MIF, macrophage migration-inhibition factor.

¶To whom correspondence should be addressed. E-mail: idit.shachar@weizmann.ac.il.

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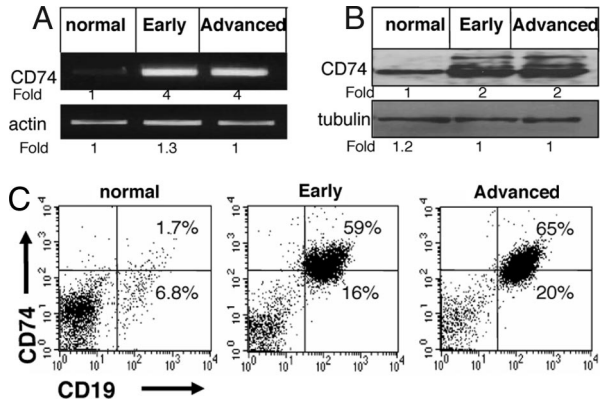

Fig. 1. Elevated expression of hCD74 in B-CLL cells. B cells derived from healthy subjects (normal), as well as early- and advanced-stage B-CLL patients, were purified. (*A*) CD74 mRNA was analyzed by RT-PCR. The results presented are representative of 8 normal, 15 early-stage, and 14 advanced-stage B-CLL patients. (*B*) Cells were lysed, and levels of the CD74 protein were analyzed by Western blot analysis. The results presented are representative of 5 normal, 11 early-stage, and 10 advanced-stage B-CLL patients. (*C*) Cells were double-stained with anti-CD19 and anti-CD74. Dot plots show CD74 expression on $CD19^+$ cells. The results presented are representative of five normal, five early-stage, and three advanced-stage B-CLL patients.

Human CD74 Activates a Cell-Survival Pathway. We demonstrated previously that in mouse B cells, CD74 releases its cytosolic domain (CD74-ICD) and initiates a signaling pathway that activates NF-κB and promotes cell survival (13). To determine whether human CD74 (hCD74) triggers a similar cascade, we followed the behavior of the p35 isoform (amino acids 1–232) in HEK-293-transfected cells. HEK-293-transfected cells were lysed with hot SDS, and the release of CD74-ICD was analyzed by Tricine gel. As seen in Fig. 2*A*, an intramembrane cleavage event liberated the CD74 p35 isoform cytosolic domain. Furthermore, CD74 cell-surface stimulation with an agonist antibody or MIF (data not shown) resulted in the specific augmentation of CD74-ICD release.

We next sought to verify whether hCD74, like its murine counterpart, can activate the NF-κB activation domain in the nucleus (9). To this end, a fusion construct containing the C-terminal transactivation domain of p65/RelA and the DNA-binding domain of the yeast transcription factor Gal4 was cotransfected into HEK-239 cells, along with a luciferase reporter containing the Gal4-binding sites, with hCD74 (9), and with the RSV promoter that was used as a reference. As shown in Fig. 2*B*, p35 induced activation of NF-κB activation domain.

To reveal whether hCD74 induces a survival cascade, we examined its effect on the transcription of Bcl-2 in HEK-239 cells. Full-length hCD74 and its cytosolic domain (amino acids 1–72) indeed induced Bcl-2 transcription in these cells, whereas hCD74 lacking the cytosolic domain (amino acids 73–232) was unable to elevate Bcl-2 mRNA levels, thereby demonstrating that the cytosolic domain of hCD74 is an essential component of the survival cascade (Fig. 2*C*). Moreover, activation of cell-surface hCD74 with an activating anti-CD74 antibody or with MIF, the natural ligand of CD74 (5), significantly elevated the levels of Bcl-2 transcription in HEK-239 cells (Fig. 2 *D* and *E*). This elevation was specific to CD74 stimulation and did not occur in cells transfected with empty construct or with hCD74 lacking its cytosolic domain (Fig. 2*E*). Thus, similar to the pathway that we recently demonstrated in mouse cells (13), human CD74 activates a cell-survival pathway resulting in Bcl-2 transcription.

Activation of Cell-Surface CD74 in B-CLL Cells Initiates a Survival Cascade. To determine whether cell-surface CD74 has a similar role in B-CLL cells and transmits a signal that results in activation of downstream signaling cascades in B-CLL cells, we used the stimulatory anti-CD74 antibody, which recognizes the CD74 extracellular domain of both the human and mouse gene products. As

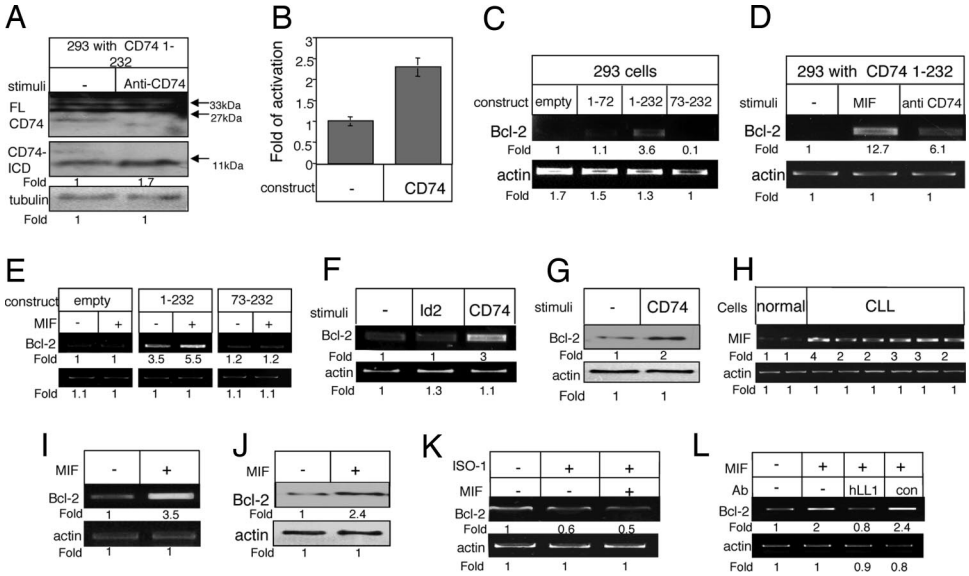

Fig. 2. MIF induces Bcl-2 expression in B-CLL cells in a CD74-dependent manner. (*A*) HEK-293 cells transfected with full-length human CD74 (amino acids 1–232) were stimulated for 30 min with or without anti-CD74 antibody. Cells were lysed by hot SDS, and the lysates were then separated on Tricine gel and analyzed with the IN1 antibody, followed by anti-rat HRP antibodies. The results shown represent three independent experiments with similar results. (*B*) HEK-293 cells were transfected with FL CD74 or with an empty plasmid. NF-κB activation was analyzed by a luciferase assay, as described in *Methods*. The results shown represent four independent experiments with similar results. (*C–E*) HEK-293 cells transfected with FL CD74 (amino acids 1–232) (*C–E*), with an empty plasmid (*C* and *E*), or with the truncated (amino acids 72–232, *C* and *E*; or amino acids 1–72, *C*) constructs. Cells were incubated in the presence or absence of anti-CD74 (*D*) or MIF (*D* and *E*) for 18 h. RNA was purified, and levels of Bcl-2 and actin mRNA were analyzed. The results shown represent three separate experiments with similar results. (*F* and *G*) B-CLL cells were incubated in the presence or absence of anti-CD74 antibody for 18 h. (*F*) RNA was purified, and Bcl-2 and actin mRNA was analyzed. The results presented are representative of 12 B-CLL patients. (*G*) Cells were lysed, and levels of Bcl-2 and tubulin were analyzed by Western blot analysis. The results presented are representative of six B-CLL patients. (*H*) RNA from B cells derived from healthy subjects (normal) and B-CLL patients was purified, and MIF and actin mRNA were analyzed. The results presented are representative of 29 B-CLL patients. (*I* and *J*) B-CLL cells were incubated in the presence or absence of MIF. (*I*) RNA was purified, and Bcl-2 and actin mRNA were analyzed. The results presented are representative of nine B-CLL patients. (*J*) Cells were lysed, and Bcl-2 and tubulin were analyzed by Western blot analysis. The results presented are representative of six B-CLL patients. (*K* and *L*) B-CLL cells were incubated in the presence or absence of MIF (100 ng/ml) (*J* and *K*), ISO-1 (20 μM) (*K*), hLL1 (50 μg/ml) (*L*), or a control antibody (*L*) for 18 h. RNA was purified, and levels of Bcl-2 and actin mRNA were analyzed. The results presented are representative of 7 (*K*) and 11 (*L*) B-CLL patients.

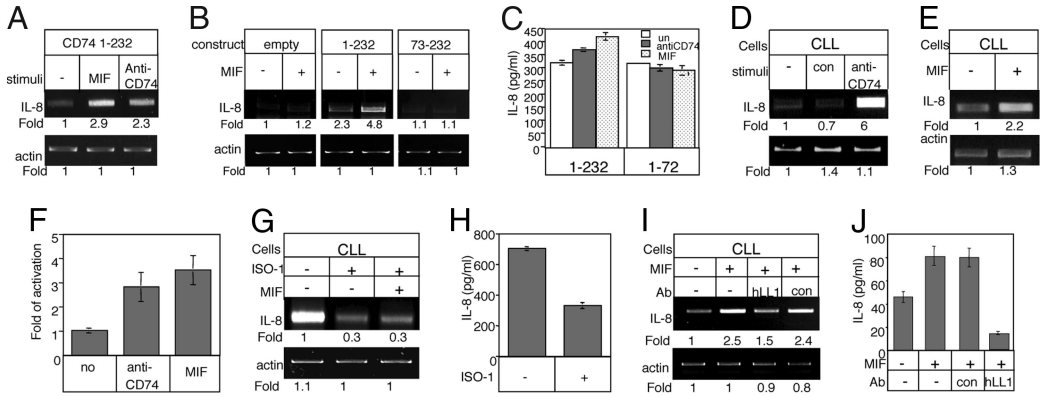

Fig. 3. CD74 induces IL-8 expression. (*A* and *B*) HEK-293 cells were transfected with an empty plasmid (*B*), with FL CD74 (amino acid 1–232) (*A* and *B*), or with the truncated (amino acid 73–232) (*B*) constructs. Cells were incubated in the presence or absence of anti-CD74 (*A*) or MIF (*A* and *B*) for 18 h. RNA was purified, and levels of IL-8 and actin mRNA were analyzed. The results shown represent three separate experiments with similar results. (*C*) HEK-293 cells transfected with FL CD74 (amino acids 1–232) or the truncated (amino acids 1–72) constructs. Cells were then incubated in the presence or absence of anti-CD74 or MIF for 18 h. The cells' conditioned medium was collected, and IL-8 levels in the conditioned medium were analyzed by ELISA. The results presented are representative of three independent experiments. (*D–F*) B-CLL cells were incubated in the presence or absence of anti-CD74 antibody (*D* and *F*); Id2, a control antibody (*D*); or MIF (*E* and *F*) for 18 h. (*D* and *E*) RNA was purified, and levels of IL-8 and actin mRNA were analyzed. The results presented are representative of 12 B-CLL patients. (*F*) Cells' conditioned medium was collected, and ELISA analyzed their IL-8 levels. IL-8 levels in unstimulated cells were normalized to 1, and the ratio for each treatment was calculated as levels in each treatment relative to 1. The results presented are representative of three independent experiments. (*G* and *H*) B-CLL cells were incubated in the presence or absence of MIF (100 ng/ml) or ISO-1 (20 μM). (*G*) RNA was purified, and levels of IL-8 and actin mRNA were analyzed. The results presented are representative of six B-CLL patients. (*H*) Cells' conditioned medium was collected, and their IL-8 levels were analyzed by ELISA. The results presented are representative of nine B-CLL patients. (*I* and *J*) B-CLL cells were incubated in the presence or absence of MIF (100 ng/ml) or hLL1 (50 μg/ml). (*I*) RNA was purified, and levels of IL-8 and actin mRNA were analyzed. The results presented are representative of 11 B-CLL patients. (*J*) Cells' conditioned medium was collected, and their IL-8 levels were analyzed by ELISA.

shown in Fig. 2, anti-CD74 stimulation up-regulated Bcl-2 mRNA (Fig. 2*F*) and protein (Fig. 2*G*) levels, indicating that activation of B-CLL CD74 initiates a survival cascade.

MIF has also been associated with the growth of malignant cells (16). To determine whether MIF expression is up-regulated in B-CLL cells, we analyzed its mRNA levels in healthy individuals (control) as well as B-CLL patients. As shown in Fig. 2*H*, a significant elevation in MIF transcription was detected in B-CLL cells, suggesting that MIF plays a role in these malignant cells. We next analyzed the effect of MIF on the survival cascade in B-CLL cells. As demonstrated in Fig. 2, MIF activation increased Bcl-2 expression (Fig. 2*I*) and protein production (Fig. 2*J*), demonstrating that MIF induces a survival cascade in these malignant cells (see SI Table 3).

ISO-1 is a nontoxic inhibitor of MIF that binds to bioactive MIF at its N-terminal tautomerase site (17). To determine whether MIF secreted from B-CLL regulates Bcl-2 expression, we analyzed its mRNA levels in cells incubated in the presence or absence of this inhibitor. As shown in Fig. 2*K*, ISO-1 down-regulated Bcl-2 mRNA levels significantly, showing that MIF secreted by B-CLL cells regulates their survival. Moreover, we used a humanized anti-CD74 antagonistic antibody (hLL1) that has demonstrated therapeutic potential on malignant cells (18). As shown in Fig. 2*L*, hLL1 specifically down-regulated Bcl-2 mRNA levels, demonstrating that blocking CD74 activity inhibits cell survival. Thus, MIF binding to CD74 induces survival of human B-CLL cells.

IL-8 Is a Target Gene of CD74 in B-CLL Cells. It was demonstrated previously that B-CLL cells constitutively produce and release biologically active IL-8. IL-8 functions to promote the accumulation of B-CLL cells by prolonging survival (19). Moreover, the bacterium *H. pylori* was recently shown to bind to CD74 on gastric epithelial cells and to stimulate IL-8 production (6). Therefore, we wished to determine whether IL-8 serves as a target gene influenced by CD74 activation in B-CLL cells and participates in the survival pathway.

To determine whether IL-8 expression is indeed modulated by CD74, we first used RT-PCR to analyze its transcription levels in HEK-293 cells transfected with full-length hCD74 (amino acids 1–232) versus an empty vector or a CD74 construct lacking its cytosolic domain (amino acids 73–232). As seen in Fig. 3 *A* and *B*, activation of cell-surface CD74 by either agonistic anti-CD74 antibody or MIF increased IL-8 mRNA levels. The elevation in IL-8 mRNA was specific to CD74 stimulation and the release of its cytosolic domain, because it was not observed in cells transfected with empty construct or with hCD74 lacking its cytosolic domain (Fig. 3*B*). We next used an ELISA to analyze IL-8 secretion after CD74 stimulation. As shown in Fig. 3*C*, stimulation of hCD74 transfected to HEK-293 cells with MIF or with anti-CD74 agonistic antibody augmented IL-8 secretion.

We next examined whether IL-8 expression in B-CLL cells is regulated by CD74. To this end, IL-8 gene expression was examined after CD74 stimulation by the agonistic antibody and was found to be augmented (Fig. 3*D*). We also examined whether MIF regulates IL-8 expression by examining B-CLL cells stimulated with 100 ng/ml MIF. As shown in Fig. 3*E*, IL-8 transcription was augmented after MIF stimulation of these cells. To further support the mRNA RT-PCR data, we used an ELISA to analyze the levels of IL-8 secretion into the conditioned medium of cells derived from B-CLL patients 18 h after CD74 stimulation (Fig. 3*F*). Approximately 2- to 4-fold increases in IL-8 secretion were detected after CD74 stimulation by either agonistic antibody or MIF (see SI Table 4) in B-CLL cells when compared with unstimulated cells. These data collectively support the role of CD74 in up-regulating IL-8 secretion in B-CLL cells.

To elucidate whether MIF secreted from B-CLL cells regulates their IL-8 expression in an autocrine manner, cells were incubated in the presence or absence of the MIF inhibitor ISO-1 (Fig. 3 *G* and *H*) or CD74 blocker hLL1 humanized antibody (Fig 3 *I* and *J*), and IL-8 expression was then analyzed. ISO-1 and hLL1 significantly inhibited IL-8 transcription (Fig. 3 *G* and *I*), resulting in a reduction of its secretion into the cell-conditioned medium (Fig. 3 *H* and *J*). Thus, MIF secreted from B-CLL cells activates CD74, resulting in regulation of IL-8 secretion.

IL-8 Induces a Signaling Cascade Resulting in Bcl-2 Expression in B-CLL Cells. In the next series of experiments, we tested whether the signal transmitted by IL-8 results in activation of downstream signaling

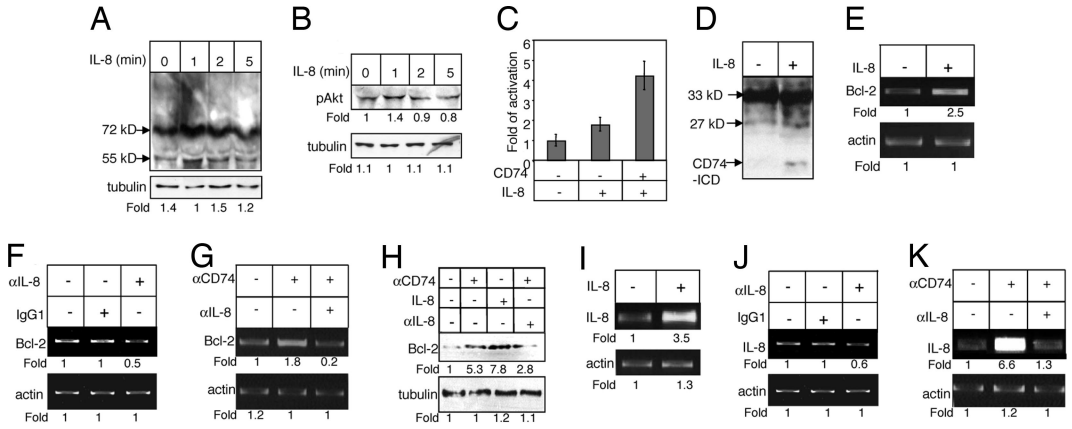

Fig. 4. IL-8, secreted after CD74 stimulation, regulates Bcl-2 expression in an autocrine manner. (*A* and *B*) B-CLL cells were incubated in the presence or absence of IL-8 (5 ng/ml) for various periods. Cells were fast-frozen and lysed as described in *Methods*. Lysates were separated on 10% (wt/vol) SDS/PAGE and blotted with anti-*p*-Tyr antibody (*A*) (results presented are representative of four separate experiments) or with anti-*p*-Akt antibodies (*B*) (results presented are representative of four separate experiments) followed by HRP-conjugated anti-mouse antibodies. The membranes were then stripped and blotted with anti-tubulin (*A* and *B*). (*C*) HEK-293 cells were transfected with FL CD74 or with an empty plasmid in the presence or absence of IL-8 (5 ng/ml). NF-κB activation was analyzed by a luciferase assay, as described in *Methods*. The results shown represent four independent experiments with similar results. (*D*) B-CLL cells were incubated in the presence or absence of IL-8 for 0.5 h. Cells were then lysed by hot SDS, and lysates were separated on Tricine gel and analyzed with anti-CD74 antibody followed by anti-goat HRP antibodies. The CD74 isoforms and the released CD74 fragment (CD74-ICD) are indicated. The results presented are representative of at least five different experiments. (*E* and *F*) B-CLL cells were incubated in the presence or absence of IL-8 (5 ng/ml) (*E*) or anti-IL-8 (10 μg/ml) or a control IgG1 antibody (10 μg/ml) (*F*) for 18 h. RNA was purified, and levels of Bcl-2 and actin mRNA were analyzed. The results presented are representative of seven B-CLL patients. (*G* and *H*) B-CLL cells were incubated in the presence or absence of anti-CD74, IL-8, or anti-IL8 for 18 h. (*G*) RNA was purified, and levels of Bcl-2 and actin mRNA were analyzed. The results presented are representative of seven B-CLL patients. (*H*) Cells were lysed, and levels of Bcl-2 and tubulin were analyzed by Western blot analysis. The results presented are representative of six B-CLL patients. (*I*) B-CLL cells were incubated in the presence or absence of IL-8 (5 ng/ml) for 18 h. RNA was purified, and levels of IL-8 and actin mRNA were analyzed. The results presented are representative of 12 B-CLL patients. (*J* and *K*) B-CLL cells were incubated in the presence or absence of anti-CD74 (*K*) or anti-IL8 (*J* and *K*) for 18 h. RNA was purified, and levels of IL-8 and actin mRNA were analyzed. The results presented are representative of six B-CLL patients.

cascades. B-CLL cells were first stimulated in the presence or absence of IL-8 for various periods, and phosphorylated proteins were analyzed by Western blot analysis by using anti-*p*-Tyr antibody. As shown in Fig. 4*A*, stimulation with IL-8 resulted in phosphorylation of mainly two bands of ≈72 and ≈55 kDa. Thus, IL-8 initiates a specific signal cascade resulting in phosphorylation of certain intracellular proteins. Because the molecular mass of Akt is ≈55 kDa, we investigated whether the PI3K/Akt pathway is activated after IL-8 stimulation. B-CLL cells were incubated for different time periods with IL-8. Next, cells were lysed and phosphorylated proteins were analyzed by Western blot analysis by using an anti-*p*-Akt antibody. The membrane then was stripped and reprobed with an anti-tubulin antibody to normalize the total protein level. Increased Akt phosphorylation was detected in B-CLL cells, peaking at 1 min after stimulation (Fig. 4*B*).

To determine whether IL-8 transmits a signal that results in activation of the activation domain in the nucleus (9), we analyzed its role in NF-κB activation in HEK-293 transfected cells. As shown in Fig. 4*C*, IL-8 induced the activation of NF-κB to a significantly higher level in the CD74-expressing cells. Thus, IL-8 activates NF-κB in a CD74-dependent manner. To clarify whether IL-8 induces a CD74 downstream cascade, we analyzed its effect on CD74 intramembrane cleavage and the release of the CD74 cytosolic domain (CD74-ICD). As shown in Fig. 4*D*, IL-8 up-regulated the release of CD74-ICD, showing that IL-8 enhances the signaling cascade induced by MIF and CD74.

To examine whether IL-8 regulates the survival pathway initiated by CD74 stimulation, B-CLL cells were first stimulated with IL-8, and the expression of the B cell antiapoptotic gene Bcl-2 was analyzed. As shown in Fig. 4*E*, IL-8 significantly elevated Bcl-2 mRNA steady-state levels. Similar results were obtained with cells from patients at all stages of the disease.

The possibility that endogenous IL-8 may mediate a survival signal in B-CLL cells was further investigated in the presence of a neutralizing anti-human IL-8 monoclonal antibody. B-CLL cells were incubated in the presence or absence of anti-IL-8 or a control antibody (IgG1). As shown in Fig. 4*F*, whereas control antibody did not affect Bcl-2 mRNA, anti-IL-8 down-regulated the mRNA levels of this gene. To further show that IL-8 regulates the CD74-induced Bcl-2 mRNA levels, B-CLL cells were stimulated with a stimulatory anti-CD74 antibody and cultured with or without the blocking anti-IL-8 antibody (10 μg/ml) for 18 h. Bcl-2 transcription levels were then analyzed by means of RT-PCR. As shown in Fig. 4*G*, stimulation with anti-CD74 antibody increased Bcl-2 mRNA levels significantly. However, addition of anti-IL-8 mAb significantly down-regulated Bcl-2 mRNA levels even less than those of untreated cells. To corroborate the mRNA RT-PCR data at the protein level, cells were lysed, and Bcl-2 protein was analyzed by Western blot analysis by using anti-Bcl-2 antibody. As shown in Fig. 4*H*, stimulation of B-CLL cells with stimulatory anti-CD74 or IL-8 resulted in overexpression of Bcl-2 protein. Addition of anti-IL-8 to the stimulated cells down-regulated Bcl-2 protein levels. Thus, CD74 initiates a signaling cascade that induces IL-8 secretion. Secreted IL-8 then further activates a survival pathway in B-CLL cells.

IL-8 Autoregulates Its Own Expression. Once we determined that IL-8 is a key protein in the CD74-induced survival cascade, we decided to track the mechanism that regulates its expression. We began by investigating the possibility that IL-8 could modulate its own expression in B-CLL cells in an autocrine fashion. To determine whether IL-8 could augment its own transcription, B-CLL cells were incubated in the presence or absence of IL-8 (5 ng/ml) for 18 h, and IL-8 transcription was then analyzed. As shown in Fig. 4*I*, IL-8 mRNA levels were elevated in cells incubated in the presence of exogenous IL-8. Blocking of IL-8 secretion by B-CLL cells specifically inhibited its own transcription (Fig. 4*J*). We next analyzed the role of secreted IL-8 in this autocrine regulation, after CD74 cell-surface stimulation. Activation of CD74 elevated IL-8 mRNA levels, a process that was inhibited by anti-IL8 mAb (Fig.

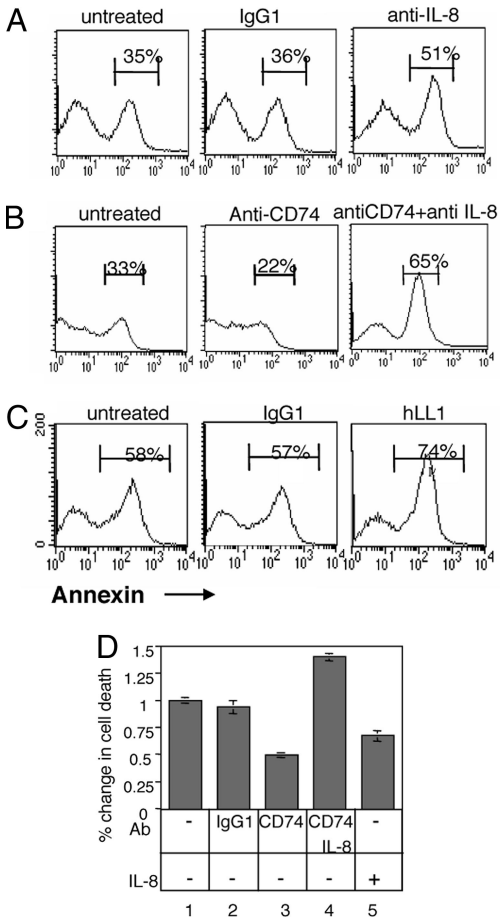

Fig. 5. IL-8 secreted after CD74 stimulation regulates B-CLL cell survival. (*A–C*) B-CLL cells were incubated in the presence or absence of anti-CD74 (*B*), anti-IL-8 (*A* and *B*), or a control antibody (c-jun; *A*) for 48 h or hLL1 and a control antibody (*C*). Cells were stained for Annexin V and analyzed by FACS. The results presented are representative of seven B-CLL patients. (*D*) B-CLL cells were incubated in the presence or absence of a control antibody (c-jun), anti-CD74, anti-IL-8, or IL-8 for 48 h. Cell death was analyzed by ELISA. The graph shows the results of one experiment, which was representative of four.

4*K*). These results suggest that IL-8 is involved, in an autocrine manner, in the regulation of its own transcription. Therefore, the CD74 cytosolic domain induces a signaling cascade that induces both IL-8 secretion and transcription of survival genes.

IL-8 Regulates B-CLL Cell Survival. Finally, to directly demonstrate whether anti-CD74 agonistic antibody or IL-8 stimulation induces B-CLL cell survival, B-CLL cells were incubated in the presence or absence of anti-IL-8 mAb and a control antibody (IgG1). The cells were then analyzed for Annexin staining. As shown in Fig. 5*A*, incubation with anti-IL-8 resulted in a specific elevation in Annexin-positive cells. In addition, as shown in Fig. 5*B*, CD74 stimulation reduced the percentage of Annexin-positive cells, whereas in cells incubated with both anti-CD74 and anti-IL8 antibodies, there was a dramatic elevation in the Annexin-positive population. Moreover, incubation with hLL1, the CD74 antagonistic antibody, significantly elevated the apoptotic population (Fig. 5*C*). Thus, CD74 regulates B-CLL survival. Whereas its stimulation reduced the apoptotic population, there was a significant elevation in this same population in the absence of IL-8.

We further followed the role of CD74 and IL-8 in B-CLL survival using a cell-death ELISA. As shown in Fig. 5*D*, incubation with IL-8 (lane 5) or anti-CD74 stimulation (lane 3) reduced cell death, a process that was specifically inhibited by anti-IL-8 mAb (lane 4) regardless of the clinical status of the patients. Thus, CD74 stimulation regulates cell death by secretion of IL-8.

Discussion

Chronic lymphocytic leukemia is a malignant disease of small mature lymphocytes, manifested by the progressive accumulation of the malignant cells, mainly due to decreased apoptosis. In this study, we have shown overexpression of CD74 in CLL cells regardless of the clinical status of the patients. We also demonstrated that CD74 expression leads to a significant augmentation of a survival cascade in B lymphocytes, and that CD74 signaling plays an important role in the increased survival of CLL cells.

In macrophages, CD74 is a high-affinity binding protein for the proinflammatory cytokine MIF (5). It is interesting to note that MIF is also related to tumor progression: MIF has also been associated with the growth of malignant cells (16, 20). In addition, anti-MIF Ig therapy has been shown to induce an antitumor response (21). We demonstrated previously that CD74 expressed on the surface of normal, mature, murine B cells initiates a signaling pathway that activates NF-κB and leads to subsequent cell survival (9, 13).

In the current study, we followed this MIF-induced CD74-mediated pathway in human B-CLL cells and analyzed the genes whose expression is induced by this activation. We demonstrate that B-CLL cells markedly up-regulate both expression of their cell-surface CD74 and their MIF production. Stimulation of CD74 with an agonistic antibody or with the MIF ligand initiates a signaling cascade leading to IL-8 transcription and secretion in all B-CLL cells, regardless of the clinical status of the patients. Secreted IL-8 induces the transcription and translation of the antiapoptotic protein Bcl-2 and thus regulates an antiapoptotic pathway, though no effect on proliferation was observed (data not shown). Blocking of CD74 (by hLL1), the secreted MIF, or the IL-8 target genes results in dramatic down-regulation of Bcl-2 expression, and augmentation of apoptosis, which is consistent with previous studies showing antiproliferative effects of hLL1 against B cell lymphomas *in vitro* and *in vivo* (18). Thus, these studies demonstrate that CD74 and its ligand, MIF, play a pivotal role in the regulation of B-CLL cell survival.

IL-8 is a member of the CXC chemokine family that plays an important role in autoimmune, inflammatory, and infectious diseases (22–24). Because of its potent proinflammatory properties, IL-8 is tightly regulated, and its expression is low or undetectable in normal tissues. However, it is now known that IL-8 also possesses tumorigenic and proangiogenic properties (25). Our studies indicate that the activation of cell-surface CD74 augments IL-8 secretion, which then regulates its own expression in an autocrine manner. We demonstrated previously that activation of CD74 leads to activation of the p65/RelA member of the NF-κB family in B lymphocytes (9, 13). Taken together, our data suggest that the CD74-signaling cascade initiates the activation of NF-κB, resulting in increased IL-8 expression and in an autocrine/paracrine survival response by B-CLL cells.

In conclusion, our data show that overexpression of CD74 in CLL is an important survival mechanism that appears to be an early event in the pathogenesis of the disease. These findings could pave the way toward unique therapeutic strategies that may be aimed at interrupting this survival pathway.

Materials and Methods

Patient Population. B lymphocytes taken from the peripheral blood of both healthy subjects (normal) and CLL patients who satisfied diagnostic and immunophenotype criteria for CLL, at various stages of the disease, were provided in accordance with the institutional review board of the hospital, as described previously (26). The diagnosis of CLL was based on standard criteria and patients were staged according to the Rai staging system (27).

Cell Purification. B lymphocytes were purified by using a RosettSep antibody mixture (StemCell Technologies, Vancouver, BC, Canada), as described previously (26). This purification had no effect on the results; the total unpurified B-CLL cells showed similar results in all of the main results presented in the manuscript (data not shown). Cells were used fresh or viably frozen in FCS plus 10% DMSO for storage in liquid nitrogen. Frozen cells were cultured overnight in 5% CO_2 in RPMI medium 1640 with 10% FCS and antibiotics. No difference in the behavior of thawed and unfrozen cells was observed.

Reagents. The parental murine anti-CD74 mAb LL1 (formerly called EPB-1) was generated by using the Raji cell line as the immunogen (28). Development of hLL1 (milatuzumab), the humanized anti-CD74 mAb, was described previously (18), and the antibody was gifted from Immunomedics (Morris Plains, NJ). Recombinant MIF was purified from an expression system as previously described and contaminating endotoxin removed by C8 chromatography (29).

Stimulation and Blocking. For MIF stimulation or blocking, cells were incubated in RPMI medium 1640 containing 0.1% (vol/vol) FCS at 37°C for 3 h.

Stimulation: 1×10^7 B-CLL cells were suspended in 1 ml of RPMI medium 1640 in the presence of 1 μg of antibody specific for the luminal domain of CD74 (C-16; Santa Cruz Biotechnology, Santa Cruz, CA) or an isotype control antibody, Id2 (Santa Cruz Biotechnology), or 100 ng/ml of MIF or recombinant human IL-8 (5 ng/ml; R & D Systems, Minneapolis, MN) at 37°C for 18 h. Immediately after incubation, the cells were washed and fast-frozen in liquid N_2.

Blocking: B-CLL (1×10^7) cells were incubated in the presence of hLL1 (50 μg/ml), 20 μM ISO-1 (CalBiochem, San Diego, CA) and incubated at 37°C for 18 h or 10 μg/ml anti-human IL-8 antibody (R & D Systems) at 37°C for 18 h.

Cell Lysis by Means of Hot SDS. Lysis of cells was performed as described previously (11).

Western Blot Analysis. Proteins were separated by SDS/PAGE, transferred onto a nitrocellulose membrane, and probed with anti *p*-Tyr (pTyr99; Santa Cruz Biotechnology), anti-*p*-Akt antibody (Cell Signaling Technology, Danvers, MA), anti-CD74 (FL-293; Santa Cruz Biotechnology) or anti-Bcl-2 (C-2; Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA) or anti-Rabbit (Jackson ImmunoResearch Laboratories). The membrane was then stripped and reprobed with anti-tubulin antibody (Sigma, St. Louis, MO), followed by peroxidase-conjugated anti-mouse (Jackson ImmunoResearch Laboratories). Tricine-SDS/PAGE 16% (wt/vol) was performed and transferred to membranes as described previously (30).

ELISA. IL-8 levels were determined by using an ELISA method according to manufacturer's instructions (BD Bioscience, San Jose, CA). Cell death was determined by using an ELISA cell-death detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions.

RNA Isolation and Reverse Transcription. Total RNA was isolated from cells by using the Tri Reagent kit (Molecular Research Center, Cincinnati, OH), according to the manufacturer's instructions. Reverse transcription was carried out by using SuperScript II RT (Invitrogen, Carlsbad, CA).

Primers that were used in PCRs were as follows: BCL-2, 5′ AGATCTCTGGTTGGGATTC 3′ CACCGAACACTTGATTCTG; IL-8, 5′ TGCCAAGGAGTGCTAAAG 3′ ATTCTCAGCCCTCTTCAAA; Actin, 5′ TGAAGTGTGACGTGGACATCCG 3′; Actin, GCTGTCACCTTCACCGTTCCAG.

Flow Cytometry. Staining of CLL cells was performed as previously described (26). The following antibodies were used: CD-19 (Miltenyi Biotec, Auburn, CA) and anti-CD74 (Santa Cruz Biotechnology). For Annexin staining, cells were incubated with Annexin (BD Biosciences) for 15 min at room temperature.

Constructs. The full-length human CD74 (p35 isoform) and the truncated 1–72 and 72–232 CD74 constructs in the pcDNA 3.1/v5-HisToPo (Invitrogen) vector were characterized previously (5).

Cell Transfection. Transfections with the full-length human CD74 (p35 isoform) and the truncated 1–72 and 72–232 CD74 constructs in the pcDNA 3.1/v5-HisToPo (Invitrogen) vector, were performed as previously described (9). A total of 5 μg of DNA was used per 10-cm^2 dish.

Luciferase Assay for Monitoring NF-κB Activation. This was performed as previously described (13).

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